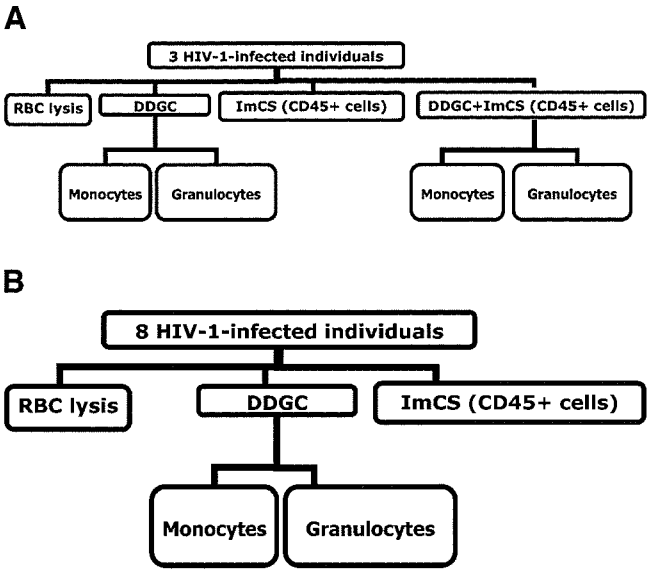

Fig. 1. Leukocyte fractionation from peripheral blood. **A**: Four different methods applied to blood of three HIV-1-infected individuals. **B**: Three different methods applied to blood of eight individuals (includes the 3 in A).

men, 1 (male) was an injecting drug user and 2 (both females) were of African origin. Nineteen patients (18%) were affected by an AIDS indicator disease, of whom five manifested KS. Fifty patients (48%) had CD counts <200 cells/μl; 39 (38%) patients were on anti-retroviral therapy. KS330 was detected from immunomagnetically fractionated CD45+ cells in 38 patients (37%); of these, 27 (71%) could be assigned to genotype A, 3 (8%) to B2, and 1 (3%) to B3/C2. Anti-HHV-8^{KS-1} was detectable in 88 patients (85%). Thirty-three of 38 (87%) anti-HHV-8^{KS-1}-positive samples were positive concordantly for KS330, this association being significant ($P < 0.001$). Both KS330 and anti-HHV-8^{KS-1} detection rates were not associated significantly with the CD4 count and the presence of AIDS indicator disease. All five of the patients with clinical KS were positive for KS330 and anti-HHV-8^{KS-1}. Anti-HSV-1 was present in 86% and anti-HSV-2 in 66%. Eighty-seven percent (75/87) of samples that were positive for anti-HHV-8^{KS-1} were concordantly positive for anti-HSV-1, but the association was not significant.

HHV-8 Genome and Herpesviral Antibody Detection in Blood Donors

The KS330 detection rate in CD45+ cells of the 100 blood donors was 8%. Six of the eight KS330 sequences could be assigned to genotype A (75%) and 2 (25%) to genotype C3. The K1/V1 detection rate was 9%; eight of the nine sequences were unique and were assignable to genotypes A1, A4, and C3 (the assignments based on ORF K1 are known not to correspond with those based on ORF 26) [Di Alberti et al., 1997]. Figure 2 depicts the diversity of the sequences relative to prototypic K1/V1 sequences. Eight of the nine K1/V1-positive samples (89%) were concordantly positive for KS330; this association was significant ($P < 0.001$). Anti-HHV-8^{KS-1}, anti-HHV-$8^{SFV/K8.1}$, anti-HSV-1, and anti HSV-2 detection rates were 24%, 12%, 28%, and 0%, respectively. No significant association was found for concordance between KS330 and anti-HHV-8^{KS-1} positivities, but the association between K1/V1 and anti-HHV-8^{KS-1} positivities was significant (OR, 4.7; 95%CI, 1.2–19.4; $P = 0.03$). There was no significant association between the positivity rate of KS330 or K1/V1 when compared against the positivity rates of anti-HHV-$8^{SFV/K8.1}$ and anti-HSV-1.

DISCUSSION

Incisive studies into the extent of infection by β- and γ-herpesviruses require implementation of approaches that facilitate the detection in body fluids of viral genomes shed not only from sites of replication but also from sites of latent infection [Wagner et al., 1992; Soderberg-Naucler et al., 1997; Martro et al., 2004]. To optimize HHV-8 genome detection in blood, we first

TABLE II. A: Results Obtained From Evaluation of Approaches to Enrich HHV-8-Carrying Blood Cells in Eight HIV-1-Seropositive Patients

Method			DDGC						DDGC + ImCS (CD45+ cells)			
Fraction	RBC lysis		Monocyte		Granulocyte		ImCS (CD45+ cells)		Monocyte		Granulocyte	
PCR	1°	2°	1°	2°	1°	2°	1°	2°	1°	2°	1°	2°
Number of positive/ number of studied	0/8	0/8	0/8	1^{c}/8	0/8	0/8	$4^{a-c,f}$/8	8/8	0/3	3^{a-c}/3	1^{b}/3	$2^{b,c}$/3

RBC, red blood cell; DDGC, double density gradient centrifugation; ImCS, immunomagnetic cell separation.
1° denotes first-round PCR; 2° denotes nested PCR.

TABLE II. B: Distribution of HHV-8 DNA in Blood Cell Subsets of Five HIV-1-Seropositive Patients

Leukocyte subset	CD45+	CD31+	CD19+	CD14+	CD2+
Number of positive/number of studied	$4^{i,j,l,m}$/5	1^{i}/5	0/5	0/5	0/5

Superscript letters denote patients designated in Table I.

TABLE III. Comparison of HHV-8 Genoprevalences, and Anti-HHV-8, Anti-HSV-1 and Anti-HSV-2 Seroprevalences Between Anti-HIV-1-Seropositive Patients and Blood Donors

Study group	Number	Age (year)	Males (%)	PCR positivity (%)		Antibody positivity (%)			
				KS330	K1/V1	Anti-HHV-8^{KS-1}	Anti-HHV-8$^{SFV/8.1}$	Anti-HSV-1	Anti-HSV-2
HIV-1-infected patients	103	41	101 (98%)	38 (37%)	n.e.	87 (85%)	n.e.	89 (86%)	67 (66%)
Blood donors	100	42	51 (51%)	8 (8%)	9 (9%)	24 (24%)	12 (12%)	28 (28%)	0

n.e., not evaluated.

evaluated the efficiency of several blood cell fractionation procedures that potentially facilitate PCR. To assure broad representation of cell types that support HHV-8 persistence, the assumption was not made that HHV-8 is exclusively carried by or tropic for any leukocytic subset, although a large body of studies has revealed HHV-8 to be particularly tropic for B cells [Blackbourn et al., 1997; Kikuta et al., 1997; Monini et al., 1999].

Four approaches that potentially facilitate HHV-8 DNA detection by nested PCR were evaluated in blood samples taken from HIV-1-seropositive patients in whom the risks of HHV-8 infection and the subsequent development of KS are high. The risk of sexually transmitted infected in these patients is also high, as indicated by the substantially higher anti-HSV-2 seropositivity rate compared to blood donors. Nonetheless, the RBC lysis approach resulted in a very low detection rate of HHV-8 DNA. This outcome may be related to the lysis of the leukocytes in the course of RBC lysis. Furthermore, the lysing procedure can be inconsistent [Pacifici et al., 1998], which would impact on PCR detection in blood of HIV-infected patients, who are prone to lymphopenia [Tiirikainen, 1995]. DDGC was considered for evaluation, to verify if granulocytes in addition to mononuclear cells provide suitable cellular substrates for PCR. Early studies seeking to optimize PCR detection of HCMV DNA and transcripts in peripheral blood had revealed the importance of sampling nucleic acids originating from cells of the granulocytic series, supplemental to those of the myeloid series [Gozlan et al., 1993]. It was observed that processing by DDGC also achieved a poor HHV-8 DNA detection rate. This outcome may be attributed to the mixture of DNA extracted from cells that carry HHV-8 with DNA in extracts from cells that do not. The protocol involving ImCS of CD45+ cells was determined to achieve the highest rates of HHV-8 DNA detection. Although the poor HHV-8 DNA detection rate achieved by the other methods may suggest a lack of specificity for the ImCS method, such a consideration does not concur with the finding that the ORF K1 sequences were almost all unique. When the ImCS protocol was applied to determining the HHV-8 genoprevalence of a larger sample of anti-HIV-1-seropositive patients, the rate obtained (37%) was what would be expected from previous studies involving HIV-1-infected populations [Humphrey et al., 1996; Dupon et al., 1997; Poggi et al., 1997].

The further ImCS study assessing which particular series of blood cells of HIV-1-seropositive patients preferentially carried HHV-8 genomes revealed that while KS330 could be detected consistently in CD45+ cells, the specific leukocyte subset that preferentially carried HHV-8 remained unidentified. KS330 was detected in the CD31+ fraction in one patient, which is consistent with previous findings implicating HHV-8 persistence in circulating KS-like spindle cell progenitors [Sirianni et al., 1997]. Why HHV-8 DNA could not be amplified from CD14+ cells is particularly puzzling,

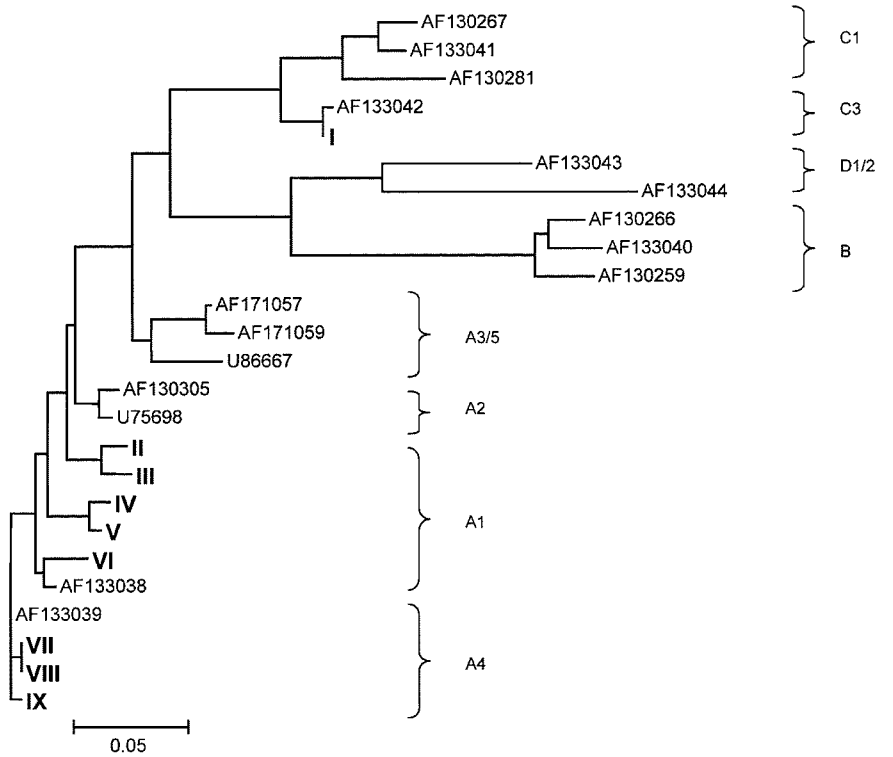

Fig. 2. Diversity and genotype distribution of K1/V1 nucleotide sequences derived from blood donors. Blood donor sequences are in bold Roman numerals. Prototypic sequences from Kaposi's sarcoma biopsy specimens bear Genbank assignment numbers. Alphanumeric assignments alongside brackets denote genotypes. Figure and horizontal bar at bottom denote proportion of nucleotides substituted for that bar length.

since a large body of studies has revealed HHV-8 to be tropic for B cells [Blackbourn et al., 1997; Kikuta et al., 1997; Monini et al., 1999]. When similar immunomagnetic fractionation procedures were applied to blood of patients who had undergone bone marrow transplantation, we were able to amplify KS330 from CD19+ cells of 40% of the patient samples (unpublished data); thus, the absence of HHV-8 genomes in CD19+ cells in the HIV-infected study group was not due to specific methodological failures.

The HHV-8 genome detection rates observed in the blood donor study sample were unexpectedly high. The KS330 detection rate (8%) may be attributed to PCR cross-contamination, but that would not explain why the KS330 sequences bear single-nucleotide polymorphisms located at known genotype-specifying base positions [Di Alberti et al., 1997; Poole et al., 1999]. Furthermore, the positivity rate for KS330 was highly concordant with that for K1/V1 (9%). Almost all the K1/V1 sequences were unique and segregated with corresponding sequences from HHV-8 strains known to be endemic to Europe [Cook et al., 1999; Zong et al., 1999]. As K1/V1 originates from a locus in the viral genome that is particularly hypervariable [Zong et al., 1999], the diversity observed in its sequences excludes the possibility of PCR contamination accounting for the high K1/V1 detection rate in the blood donor samples. The K1/V1 PCR and sequencing data, coupled with the high concordance between the KS330 and K1/V1 positivity rates, establish firmly the HHV-8 genoprevalence rate in the blood donor group to approximate 8–9%.

Estimations of the extent of HHV-8 infection in populations not at risk of KS are unreliable due to variation in the sensitivity and specificity of anti-HHV-8 antibody assays. In American blood donors, anti-HHV-8 seroprevalences have been observed to range from 0% to 23% [Lennette et al., 1996; Smith et al., 1997; Hudnall et al., 2003]. One study evaluating seven assays for anti-HHV-8 IgG showed essentially no agreement in the results obtained among the 85 sera originating from American blood donors [Rabkin et al., 1998]. A more recent study involving six laboratories each testing 1,000 American blood donors found that only 3.3% of donor plasma samples were positive for anti-HHV-8 IgG in two or more laboratories [Pellett et al., 2003]. In the northern European context, an early HHV-8 seroprevalence study of British blood donors reported a 3% rate [Simpson et al., 1996], which contrasts with the study of Enbom et al. [2000] reporting 20% in Swedish blood donors. While the latter rate matches the rates obtained in our blood donor group, the low concordances in the results obtained between HHV-8 DNA detection and our antibody assays, and between our two antibody assays, point to the poor specificity of these assays, suggesting that the specificity of the PCR method cannot be validated by current antibody assays. The alternative approach, as used in this study, is to use ORF K1 sequencing to confirm specificity.

In view of the continuing uncertainty over results obtained from anti-HHV-8-based studies of populations that are at low risk of KS, the findings from our study showing unexpectedly high HHV-8 genoprevalences in blood donors require further confirmation, particularly as evidence gathers revealing that HHV-8 may be transmissible via blood transfusion [Dollard et al., 2005].

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Original article

Pathogenesis of cytomegalovirus-associated labyrinthitis in a guinea pig model

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Abstract

Cytomegalovirus infects fetuses through the placenta, resulting in various congenital disorders in newborns, including hearing loss. We developed a monoclonal antibody to guinea pig cytomegalovirus (GPCMV) that was available for immunohistochemistry, and investigated the expression of the GPCMV antigen in animal models of direct and congenital infections. Injection of GPCMV, directly to the inner ear, increased the sound pressure level and resulted in labyrinthitis with severe inflammation. Immunohistochemistry detected GPCMV-infected cells mainly in the scala tympani, scala vestibule and spinal ganglion, but rarely in the cochlear duct. Injection of GPCMV to 5-week pregnant guinea pigs resulted in severe labyrinthitis in fetuses. Immunohistochemistry detected GPCMV-infected cells in the perilymph area and spinal ganglion, but not in the endolymph area, including hair cells. These data suggest that the virus spreads via the perilymph and neural routes in the inner ear of both models of direct and congenital infections.

Keywords: Animal model; Guinea pig; Cytomegalovirus; Hearing loss; Immunohistochemistry; Labyrinthitis

1. Introduction

Human cytomegalovirus (HCMV) is the most common cause of congenital virus infection [1]. HCMV is detected in less than 1% of all neonates, and in Japan 14% of HCMV-positive neonates were symptomatic at birth [2,3]. HCMV-associated diseases in fetuses range in severity from asymptomatic viremia to fatal encephalitis with cytomegalic inclusion bodies [1,4]. Hearing loss is one of the most common illness associated with HCMV. Ten to twenty percent of infants congenitally infected with HCMV have varying degrees of hearing loss at birth [2,3,5]. Since numerous reports have demonstrated that HCMV is detected in human placentas, transplacental transmission is common from the mother to the fetus, in humans [4,6–11]. Some studies have also reported the presence of HCMV in the human fetus or neonatal samples [12–14]. A PCR study demonstrated that HCMV can be detected in the perilymph of patients with sensorineural hearing loss [14]. A pathological study on an autopsy case of a child with acquired HCMV infection showed that cytomegalic bodies were present in the inner ear, i.e. epithelium of the endolymphatic sac, the utricle and the semicircular canals; however, no HCMV antigen was detected by immunohistochemistry [13]. Thus, although it is clear that HCMV infection is implicated in the pathogenesis of congenital hearing loss [15], the exact mechanism of congenital hearing loss still remains unclear.

Since guinea pigs (GPs) have a relatively short pregnancy period (10 weeks) and the structure of its placenta is similar to that of human, GPs have been used as an animal model

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for the investigation of vertical infection of CMV [16–20]. From the 1970's to 1980's, numerous studies were performed using GP models [20]. These studies demonstrated; (i) the GP model for CMV infection contains intrauterine infections, which cross from the mother to fetus, whereas mouse CMV did not cross the placenta or cause congenital infection; (ii) labyrinthitis could be induced by both transplacental pathways and injection of GPCMV into the inner ear; (iii) direct injection of GPCMV to the inner ear of adult GPs results in hearing loss; and (iv) anomalies in the cochlea were found in some offspring of the vertical GPCMV infection model [8,9,11,16–21]. In addition, pathological findings in the GPCMV-injected GP and the GPCMV-infected fetus are: (i) severe hemorrhage and marked inflammatory cell infiltration with cytomegalic inclusion bodies in the perilymph region and spinal ganglion; (ii) marked fibrosis in the middle ear; and (iii) utriculus, sacculus, endolymph sac and Corti organ in the cochlear duct were atrophic, but showed no GPCMV infection [8–11,22,23]. These data indicate that CMV infects the fetus through the placenta, and spreads to the inner ear via the perilymph and the spinal ganglia. However, as serum from GPCMV-infected GP was used as an anti-GPCMV antibody [22,23], and that these studies were performed before the development of sensitive immunohistochemistry methods, the specificity and sensitivity were insufficient for determination of the precise localization of GPCMV antigens. Importantly, the detailed localization of GPCMV-infected cells in the inner ear, especially in the cochlea, remains unknown in a model of vertical transmission.

Here, we performed two sets of animal experiments. First, to observe the direct effects of GPCMV infection in the inner ear, we injected GPCMV directly into the inner ear of GPs. Second, to observe the effect of GPCMV infection on the fetus, we inoculated pregnant GPs with the virus. In the present study, we demonstrate the localization of GPCMV-infected cells in samples obtained from these two experiments by immunohistochemistry using an anti-GPCMV monoclonal antibody (MAb).

2. Materials and methods

2.1. Antibody

A mouse MAb to GPCMV was developed as described previously [24]. Briefly, six-week-old BALB/c mice were immunized with GPCMV strain 22122-infected GP embryonic cells. GPCMV was kindly provided by Dr. J.P. Harris, the University of California [10]. Spleen cells were fused with myeloma (P3-NS/1-Ag4-1) cells. Hybridoma cells were selected and cloned. Finally, acites containing the antibody were collected from the peritoneal cavity of BALB/c mice injected with the cloned hybridoma cells.

2.2. Immunofluorescence assay

GP lung fibroblast (GPL: American type culture collection, Manassas, VA) cells were infected with GPCMV at an MOI of 1 in the absence or presence of phosphonoacetic acid (PAA) and cultured for 4–72 h. Immunofluorescence assays were performed as described previously [24]. MAb g-1 and fluorescein isothiocianate (FITC)-conjugated anti-mouse IgG (DAKO, Copenhagen, Denmark) were used as primary and secondary antibodies, respectively.

2.3. Real-time PCR

GPCMV DNA copy numbers were measured by real-time PCR using the primers for the GPCMV GP83 gene with a 6-carboxyfluorescein (FAM) probe (5′-FAM-ATCCGAGTTAGG CAGCG-MGB (minor groove-binding molecule)-3′) [25]. To obtain the GPCMV DNA copy numbers in a single cell, a copy number of the GP cellular gene, GP-short interspersed elements (SINE) [26], was determined by real-time PCR.

2.4. GPs and virus preparation for animal experiments

For the experiment of direct injection of GPCMV into the inner ear, two sets of 8 GPCMV-seronegative GPs of the Hartley strain of 200 g-body weight were used. One set was used for examination of auditory brainstem response (ABR), and the other for pathological examination. For the vertical infection model, we used 20 pregnant (at 3 or 5 weeks) GPCMV-seronegative GPs. GPCMV stocks for the animal models were prepared as described previously [11]. Briefly, the lysate of salivary glands was obtained from GPCMV-infected GPs. Lysates were adjusted to 1×10^6 TCID50/ml in 10% dimethylsulfoxide/Hanks solution. All animal procedures were approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases, and were conducted according to 'the Guidelines for Animal Experiments Performed at the National Institute of Infectious Diseases'.

2.5. Direct injection of GPCMV to the inner ear

The right middle ear was opened using a surgical knife [11]. In the experimental group (4 GPs), 2 μl of GPCMV (2×10^3 TCID50) was injected into the round window of the inner ear using 27 gauge needles. In the control group (4 GPs), 10% dimethylsulfoxide/Hanks solution was inoculated. The opened ears of both groups were covered with gel foam after injection. For pathological examinations, another set of GPs were sacrificed under anesthesia, at 9–12 days after the injection.

2.6. Injection of GPCMV to pregnant GPs

5×10^5 TCID50/0.5 ml of GPCMV was injected into the subcutaneous region on the back of 3 or 5 week-pregnant GPs [8,9,20]. After 3 weeks incubation or before birth, GPs were sacrificed under general anesthesia.

2.7. Auditory brainstem response

ABR was performed, as described previously, to examine the effects of virus infection in the inner ear [27]. Needle

electrodes were placed subcutaneously in anesthetized GPs. The reference electrode was inserted beneath the pinna of the measured ear, the ground beneath the opposite ear, and the active electrode beneath the skin on the top of the head. Responses for 1024 sweeps were averaged at each intensity level near the threshold, in 5 dB sound pressure level (SPL) steps. The threshold was defined as the lowest intensity level at which a clear waveform was visible in the evoked trace and was determined by visual inspection of the responses.

2.8. Pathological examination

All organs obtained from sacrificed GPs, including the fetuses, were fixed in 10% buffered formalin. Hard tissues, containing bone, were decalcified in 10% ethylenediamine tetraacetic acid (EDTA) for 2 weeks. Formalin-fixed specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, as described previously [11].

2.9. Immunohistochemistry

Immunohistochemistry was performed with MAb g-1 as the primary antibody. For the second and third phase immunostaining reagents, a biotinylated F(ab')2 fragment of rabbit anti-mouse immunoglobulin (DAKO) and peroxidase-conjugated streptavidin (DAKO) were used. 3-3'diaminobenzidine was used as a chromogen and slides were counterstained with hematoxylin.

3. Results

3.1. Characterization of an anti-GPCMV mouse MAb, g-1

To obtain an anti-GPCMV antibody that is available for immunohistochemistry, five clones of hybridomas developed in the previous study (clones B16, B-29, D-13, E-16, g-1) [24] were screened by immunohistochemistry. One of them, clone g-1, specifically labeled GPCMV-infected cells in paraffin-embedded samples. Western blotting demonstrated that the MAb g-1 recognized a 50 kD protein that was expressed from 24 h post infection (p.i.) in GPCMV-infected cells but not in mock-infected cells (Fig. 1A). We also confirmed that PAA treatment did not abolish the 50 kD protein (data not shown). IFA revealed that the MAb g-1-specific fluorescence was detected in the nucleus of GPCMV-infected GPL cells (Fig. 1B) but not in the mock-infected cells (data not shown) from 24 to 72 h p.i. Treatment with PAA did not inhibit expression of the MAb g-1-specific nuclear protein in GPCMV-infected cells (Fig. 1B). GPCMV DNA synthesis initiated at around 48 h p.i., but was completely abolished in the presence of PAA (Fig. 1C). Taking into account the timing of DNA replication, it is likely that the 50 kD viral protein is an antigen that is expressed during an early phase of the infection, which is similar to that reported with MAb E-16 [28].

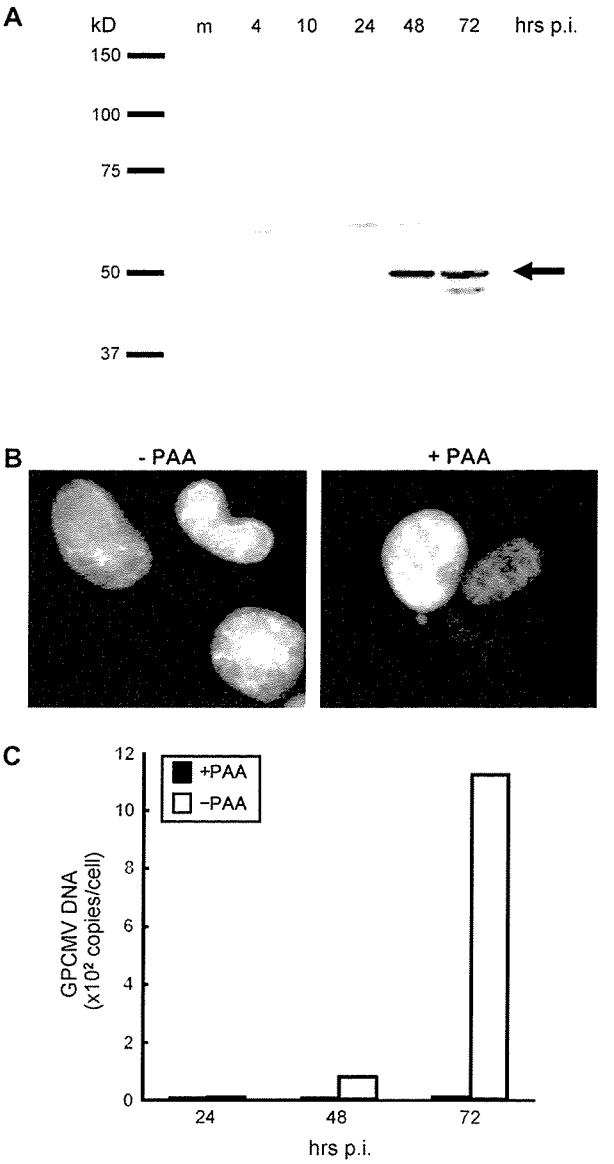

Fig. 1. Characterization of anti-GPCMV MAb g-1. (A) Expression kinetics of GPCMV protein that reacted with MAb g-1. GPL cells were mock-infected (m) or infected with GPCMV at an MOI of 3 and incubated at 37 °C (4, 10, 24, 48, 72 h p.i.). Cell lysates were analyzed by Western blotting using a MAb against GPCMV, g-1. The arrowhead indicates the protein of interest. Molecular weight standards are shown on the left of the blot. (B) MAb g-1 reacted with nuclear viral protein. GPL cells were infected with GPCMV at an MOI of 1, and incubated in the absence or presence of PAA (100 μg/ml). At 72 h p.i., GPCMV-infected cells were fixed with cold acetone, and analyzed using an indirect immunofluorescence assay (IFA). (C) GPL cells were infected with GPCMV at an MOI of 1, and incubated in the absence or presence of PAA. Total DNA was extracted from the cells at the indicated time points. Copy numbers of GPCMV and cellular DNA were measured by real-time PCRs to obtain GPCMV DNA copy numbers per cell.

3.2. Direct injection of GPCMV in the inner ear induced hearing loss

GPCMV was injected into the inner ear through the round window in four GPs. Sample buffer was injected into a further 4 GPs, as a control. We investigated baseline-hearing thresholds using ABR (Table 1 and Fig. 2). The mean of sound

Table 1
Hearing threshold

	No.	pre-operation	1 weeks	2 weeks	3 weeks
Control group	1	60	60	50	50
	2	55	90	90	90
	3	45	>100	>100	90
	4	50	>100	100	100
Experimental group	1	45	>100	>100	>100
	2	55	>100	>100	>100
	3	50	>100	>100	>100
	4	55	65	95	>100

unit: dB SPL.

pressure level (SPL) before the operation was 52.5 dB. One week after inoculation of virus, SPL went up to 60–90 dB or >100 dB even in the control group. Three weeks later, GPs in the control group showed SPL less than 100 dB, suggesting a recovery from the hearing loss. However, SPLs of all GPs in the experimental group were >100 dB, indicative of hearing loss. These data suggest that injection of GPCMV directly into the inner ear induced hearing loss in GPs at least until 3 weeks after injection.

3.3. Virus spreads in the inner ear of GPCMV-injected GPs

GPCMV-injected GPs were sacrificed 9–12 days after injection. Macroscopically, petechial hemorrhage was observed in the inner ear in the experimental group, but not in the control group. Histologically, there was marked bleeding with inflammation in the labyrinth, especially in scala vestibule, scala tympani, and spiral ganglion of the cochlea (Fig. 3A). Utriculus, sacculus, and semicircular ducts were atrophic, and slight inflammatory cell infiltration and bleeding were found in the perilymph region. In the cochlear duct, the tectrial membrane was swollen and was sometimes seen to be attached to Reissner's membrane. Immunohistochemistry revealed that there were GPCMV-infected cells in the scala vestibulli and scala tympani (Fig. 3B). In the stria vascularis, severe congestion was observed, but GPCMV antigen was not detected (Fig. 3A,B). On the surface of Reissner's membrane, GPCMV-infected cells were detected only on the side of the scala vestibulli, but not of the cochlear duct (Fig. 3C). Severe bleeding was found mainly in the scala tympani showing cytomegalic change (Fig. 3D), whereas the spiral limbus and Corti organ remained relatively intact. GPCMV-infected cells were also detected in the spinal ganglion with destruction (Fig. 3E,F). No GPCMV-antigen was detected in the utriculus, sacculus, semicircular ducts, and endolymphatic sac. Immunohistochemistry revealed that all GPs in the experimental group demonstrated similar localization of GPCMV in the inner ear. Thus, these data suggest that GPCMV spreads on the perilymph and spinal ganglion in GPCMV-injected GPs. Apart from the inner ear, GPCMV-infected cells were detected in the middle ear, salivary gland, kidney, pancreas, chroid plexias in the brain, and cerebellum (Fig. 3G,H). No GPCMV infected cells were found in the control group.

3.4. Animal model of vertical transmission

To understand the mechanism of vertical transmission from the mother to the fetus, we initially injected GPCMV into the subcutaneous region of 10 pregnant GPs at 3 weeks of gestation. Three weeks after injection, all mothers, including fetuses, were sacrificed and examined pathologically. One of 10 mothers had a generalized GPCMV infection. Thirty two fetuses were obtained from the 10 mothers. Five fetuses (15.6%) from 3 mothers were dead, while 3 (9.4%) from 2 mothers showed significantly smaller sizes than others, suggesting delayed development. In the mother with generalized CMV infection, severe degenerative necrosis, caused by the GPCMV infection, was histologically observed in the inner ear. No remarkable changes were found in other mothers from the HE stain. However, immunohistochemistry revealed

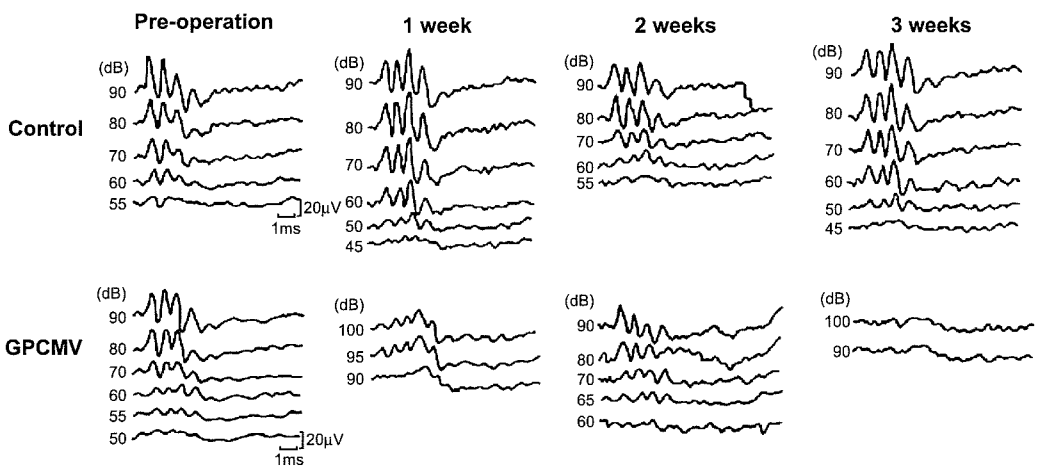

Fig. 2. Profiles of auditory brainstem response (ABR). Control: Buffer-injected GP; GPCMV: GPCMV-injected GP. In pre-operation, both control and GPCMV-infected GP showed clear waveforms in >60 dB. Waveforms disappeared completely by 3 weeks after operation in the GPCMV-infected GP (lower panels), whereas the control GP showed clear waveforms at >50 dB (upper panels).

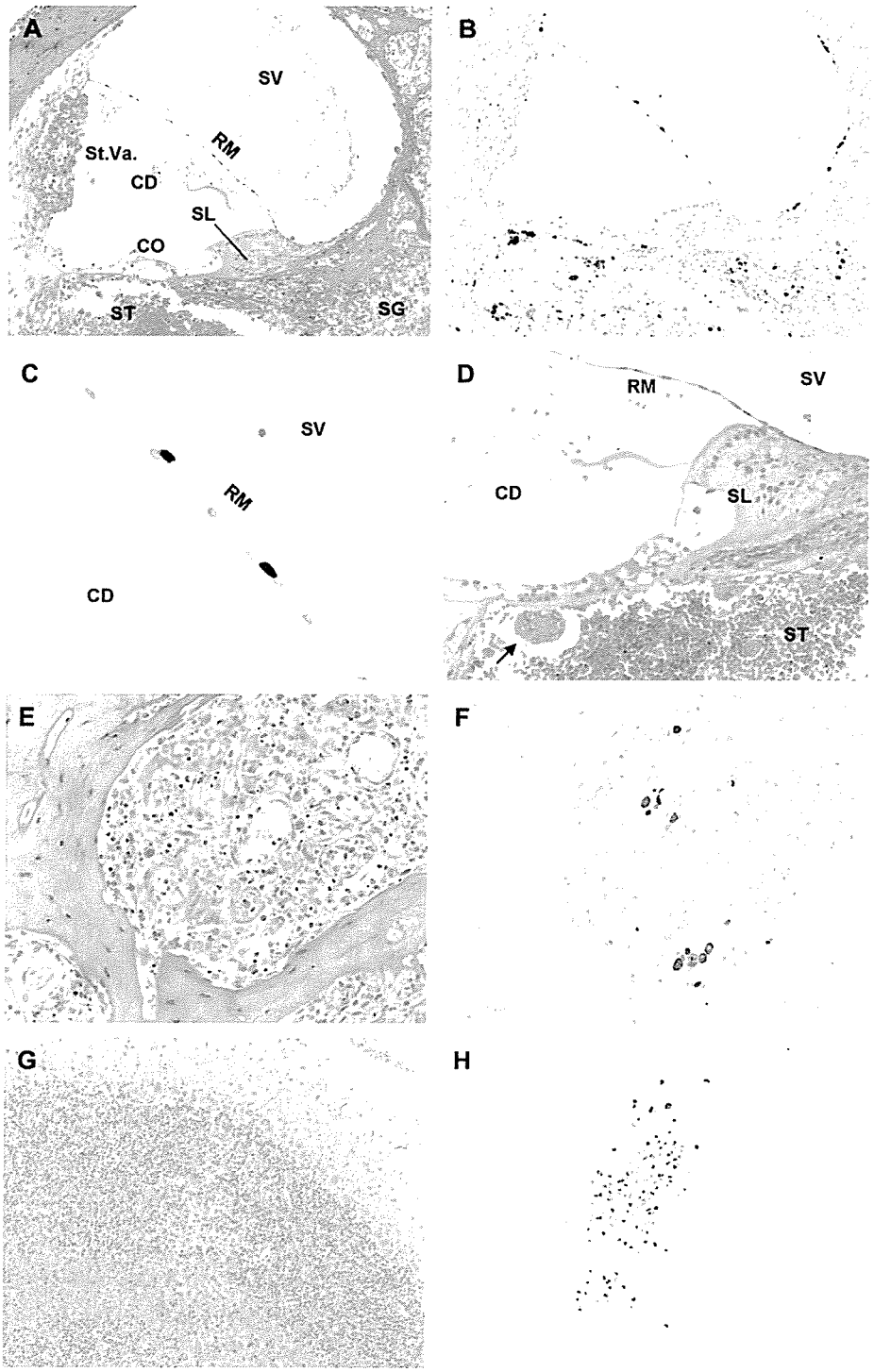

Fig. 3. Histology of guinea pigs (GPs) inoculated with GPCMV in the inner ear. CD: cochlear duct, CO: Corti organ, RM: Reissner's membrane, SL: spiral limbus, SG: spiral ganglion, ST: scala tympani, St. Va.: stria vascularis, SV: scala vestibuli. (A) Low magnification view of the cochlea. Bleeding and inflammatory cell infiltration are evident with HE staining. (B) Immunohistochemistry for GPCMV on a serial section of the panel (A). GPCMV antigens were detected predominantly in the scala vestibuli, scala tympani, and spiral ganglion, but not in the stria vascularis, Corti organ, and spiral limbus in the cochlear duct. (C) High power view of Reissner's membrane. GPCMV-infected cells were detected only on the side of the scala vestibuli, but not of the cochlear duct by immunohistochemistry. (D) High power view of the spiral limbus of another GP. Giant cells with remarkable intranuclear inclusion body are found in the scala tympani (arrow). Marked bleeding was also observed with inflammatory cell infiltration in the scala tympani. (E) HE staining of the spiral ganglion. Severe inflammation with bleeding is observed. (F) Immunohistochemistry for GPCMV on a serial section of panel (E). GPCMV antigen was detected in the ganglion. (G) Cerebellum of GPCMV-injected GP. Loss of cerebellar granule cells is found. (H) Immunohistochemistry for GPCMV on a serial section of panel (G). GPCMV antigen was detected at the site of the loss.

that GPCMV-infected cells were present in the spleen, liver, and placenta of the mothers (Fig. 4). The numbers of GPCMV-positive cells, especially in the placenta, varied among the mothers. We also investigated the inner ears of the fetuses; however, since these inner ears were immature at 6-weeks pregnancy, it was difficult to observe any changes.

We then injected GPCMV into the subcutaneous region of 10 pregnant GPs at 5 weeks of gestation. Five weeks later, the GPs, including fetuses, were sacrificed before birth. All 30 fetuses were alive. Macroscopically, petechial hemorrhage was observed in the inner ear in 4 fetuses from 2 mothers. Histological examination demonstrated various changes in the inner and middle ears of the fetuses. Remarkable changes were cytomegalic cells attaching at Reissner's membrane, necrosis in the spiral ganglion, and vacuolar degeneration of stria vascularis in the inner ears of the fetuses (Fig. 5A–F). These changes were also observed in some other fetuses, but were milder. Utriculus, sacculus, and semicircular ducts seemed atrophic in almost all fetuses. Immunohistochemistry revealed the presence of GPCMV-infected cells in the four fetuses with severe histological changes. GPCMV-infected cells were detected in the scala vestibule, scala tympani, and spiral ganglion, but not in the endolymph area such as in the cochlear duct, utriculus, sacculus, and semicircular ducts (Fig. 5B,D,F). The GPCMV antigen was detected not only in the inner ear, but also in the middle ear and kidney (Fig. 5G,H). Thus, the localization of GPCMV-infected cells in fetuses was similar to that in GPs injected with GPCMV into the inner ear. These data suggest that GPCMV was transmitted from mother to fetus through the placenta, and spread in the inner ear of the fetus through the perilymph and spinal ganglion.

4. Discussion

In the present study, we demonstrated the localization of GPCMV antigen in experimental models of vertical and horizontal GPCMV-infection, using GPs. Immunohistochemistry, using MAb g-1 to GPCMV, demonstrated that GPCMV-infected cells were detected in the scala vestibule, scala tympani, and spiral ganglion of the cochlea, but not in the cochlear duct of virus-injected GPs and fetuses with vertical GPCMV infection. The localization of GPCMV-infected cells in fetuses was similar to that in GPs injected with GPCMV into the inner ear. This is the first report detecting GPCMV antigens in the GP fetuses by immunohistochemistry using MAb. These data suggest that the GPCMV infection can spread through the perilymph and spinal ganglion in the inner ear of both experimental models.

In essence, our observations in the present study are consistent with previously reported results [8–11,22,23]. However, another group examined over 190 fetal GPs after infection of pregnant animals and failed to detect any GPCMV antigen in the labyrinth [29]. The results in that report seem to be very different from those of ours and other groups [11,19,20], despite that the authors in the report injected GPCMV at various stage of pregnancy [29]. As we demonstrated in the present study, GPCMV infection shows different symptoms depend on the timing of inoculation [17]. In addition, a formic acid and sodium citrate solution was used in the study as a decalcification solution that dramatically reduces the sensitivity of immunohistochemistry. Thus, we presume that such a methodology produced different results from ours.

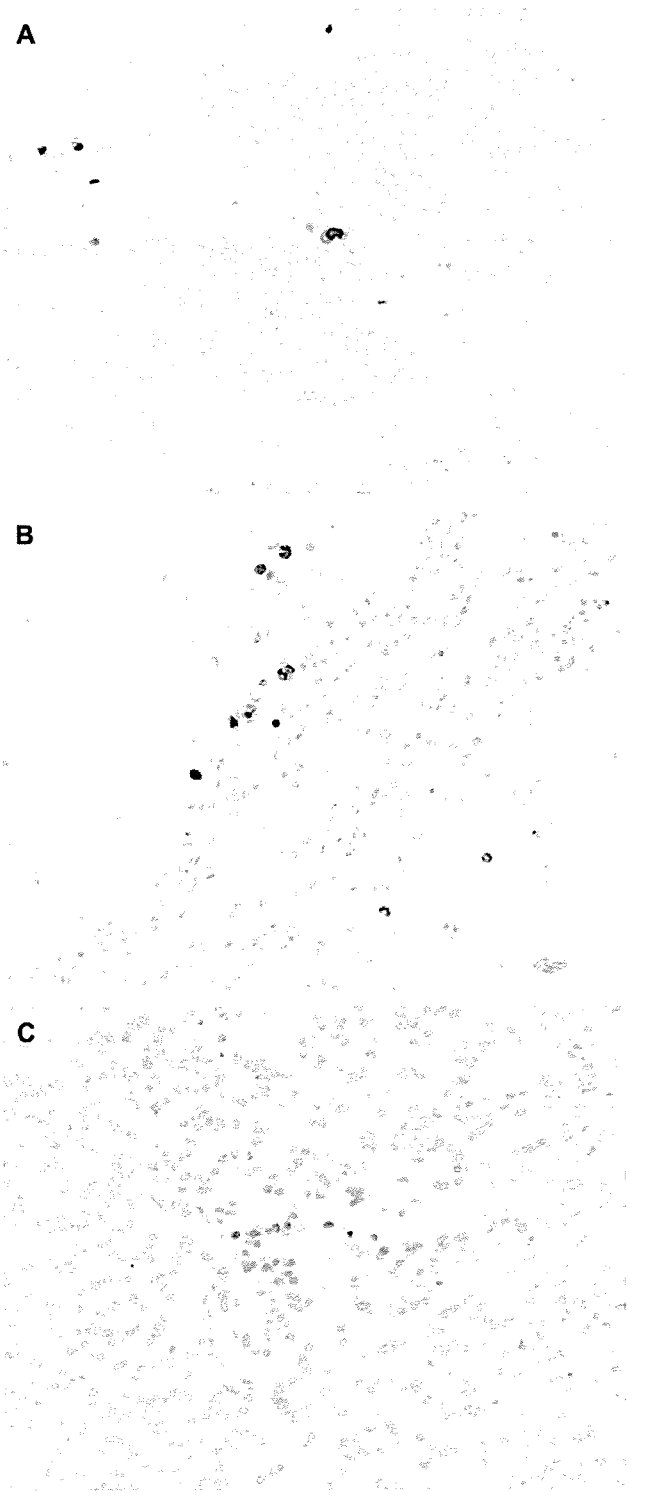

Fig. 4. Immunohistochemistry of GPCMV on organs derived from GPCMV-injected mothers. GPCMV antigen was detected in the spleen (A) liver (B), and placenta (C).

The anti-GPCMV MAb g-1 is available for immunohistochemistry, and showed clear localization of GPCMV-infected

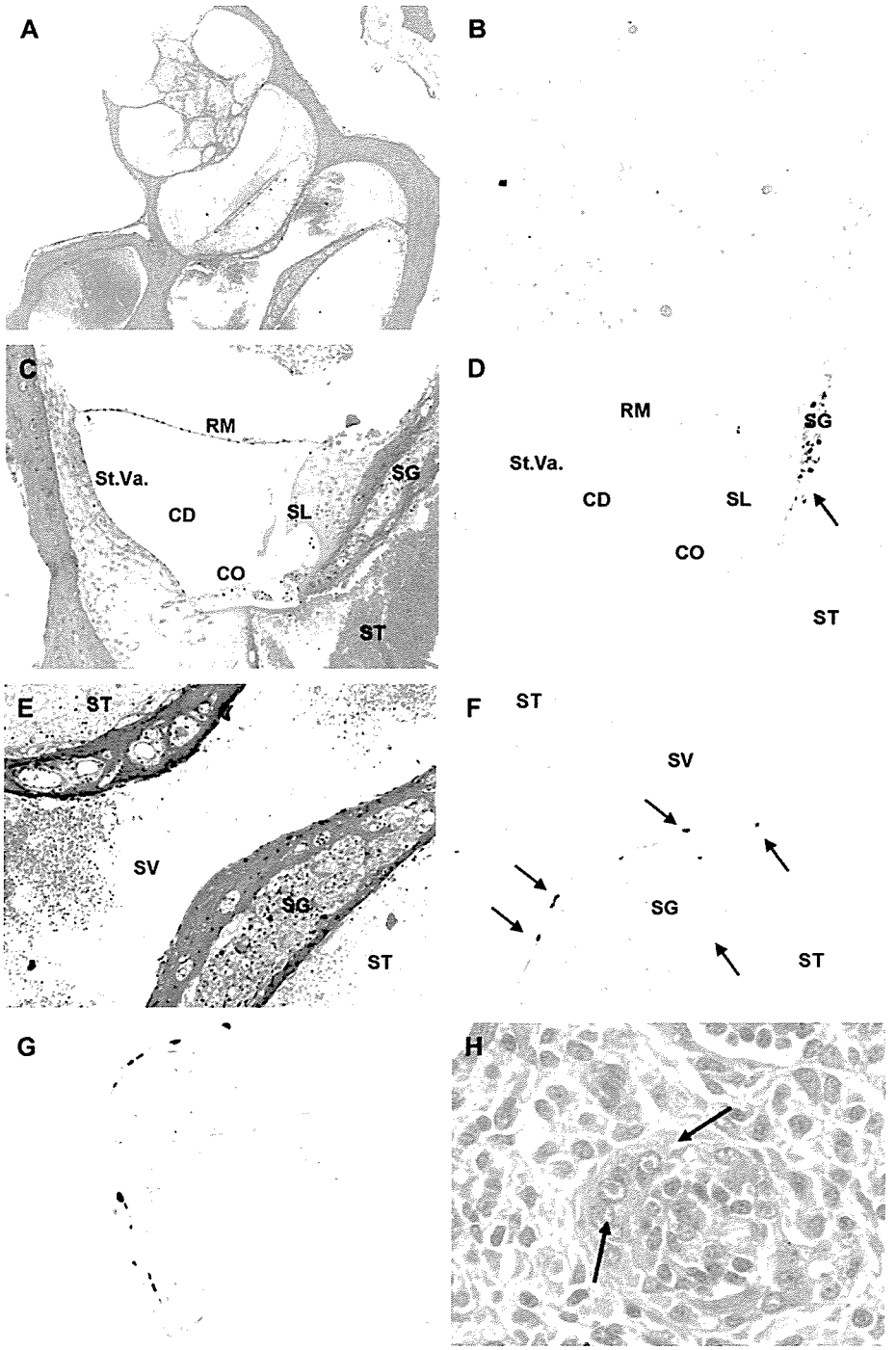

Fig. 5. Histology of the inner ear in a fetus of a GPCMV-injected mother. CD: cochlear duct, CO: Corti organ, RM: Reissner's membrane, SL: spiral limbus, SG: spiral ganglion, ST: scala tympani, St. Va.: stria vascularis, SV: scala vestibuli. (A) Low power view of the cochlea. Severe bleeding and inflammation was observed in the cochlea. (B) High power view of GPCMV-infected cells in the spinal ganglion. GPCMV-infected cells are found with severe destruction. (C) High power view of the cochlear duct of the fetus. Severe bleeding was observed in the scala tympani. (D) Immunohistochemistry revealed that GPCMV-infected cells are present in the spinal ganglion on a serial section of (C). (arrow) (E) High power view of the scala tympani, vestibuli and spinal ganglion. Marked bleeding was found in the scala tympani and vestibuli. (F) Immunohistochemistry for GPCMV in a serial section of (E). GPCMV antigen was detected in the surface membrane of the scala tympani and vestibuli, and spinal ganglion (arrows). (G) Stapes with edema and inflammatory cell infiltration. GPCMV-infected cells were detected on the surface of the stapes. (H) Cells with cytomegalic inclusion bodies were found in the kidney (arrows).

cells. The results of immunohistochemistry in the present study appear similar to the localization of cytomegalic cells in hematoxylin and eosin staining. Western blotting and IFA suggest that MAb g-1 recognizes early proteins encoded by GPCMV. Since early proteins are expressed in the early phase of infection, the antibody may fail to detect some GPCMV-infected cells in different phases of infection. However, immunohistochemistry using the MAb g-1 is much more sensitive

than cytomegalic cells for determining the localization of GPCMV-infected cells. Indeed, in the present study we observed GPCMV-positive cells, even in areas without cytomegalic cells. Thus, the present study has more accurately demonstrated the localization of CMV-infected cells than has previously been reported.

CMV may infect the inner ear through three routes; tympanogenic, meningogenic and hematogenous. Previous studies suggest that the hematogenous route may be the primary one for invasion to the inner ear [11]. Immunohistochemistry, using a MAb to GPCMV in the present study, demonstrated that the histological localization of GPCMV-infected cells in the inner ear in the vertical infection model was very similar to those of inner ear GPCMV-injected GPs. This suggests that the route of GPCMV infection in the inner ear may be similar in these two models. Interestingly, GPCMV antigen was not detected in the stria vascularis regardless of the severe congestion in GPs injected with GPCMV into the inner ear (Fig. 3A,B). Moreover, GPCMV antigen was detected in cells on the surface membrane of the staples bone in the middle ear, even in the vertical infection model (Fig. 5G). GPCMV-positive neural cells were found in the spinal ganglion of both models. Thus, these data suggest that GPCMV might spread through perilymph and ganglion in the inner ear, although GPCMV reaches the inner ear through a hematogenous route. Since no GPCMV-infected cells were detected in the endolymph area, endolymph was virus-free in both models. No virus antigen was detected in the Corti organ including outer and inner hair cells in any model. Damage to vessels around the Corti organ would affect the function of the inner and outer hair cells by peripheral circulatory disturbance. However, it is important that hair cells were not found to be damaged in any samples. These observations suggest that improvement of circulation around the Corti organ and repairing neurons in the ganglions might be an effective method for the recovery of hearing function in CMV-associated hearing loss. Moreover, these data suggest that late-onset hearing loss might be associated with peripheral circulatory disturbance by CMV reactivation in the inner ear in human. Our findings should be a useful guide for further research into gene therapies. Indeed, recent gene therapies for deafness have targeted the hair cells of the Corti organ [30]. However, judging by our results, hair cells may not be an appropriate target for CMV-associated hearing loss. Further studies using animal models will be required to clarify the mechanisms involved and to develop new treatments for CMV-associated hearing loss.

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Congenital Cytomegalovirus Infection Diagnosed by Polymerase Chain Reaction With the Use of Preserved Umbilical Cord in Sensorineural Hearing Loss Children

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Objectives/Hypothesis: **Congenital cytomegalovirus (CMV) infection is estimated to account for 30% of sensorineural hearing loss (SNHL) cases. Differences in clinical characteristics between CMV-related and unrelated SNHL cases were scrutinized.** ***Methods:*** **Using dried umbilical cord, we have recently developed a polymerase chain reaction (PCR)-based assay for the retrospective detection of congenital CMV infection. Medical records of 7 CMV-related patients identified from 31 SNHL patients by the assay were evaluated for the following: type and degree of hearing impairment, computed tomographic scan results, mental retardation, cerebral palsy, autism, and other multiple disorders.** ***Results:*** **Clinical characteristics of the seven CMV-related SNHL cases were as follows: 1) six of the seven exhibited severe bilateral SNHL, whereas one had severe unilateral SNHL in the right ear. Although the hearing levels of CMV-related patients were more greatly impaired than those of CMV-negative patients, there was no hearing impairment pattern specific to the CMV-related patients; 2) five patients had mental retardation, which was more frequent than in CMV-negative patients; 3) birth weights of the CMV-positive cases were relatively lower.** ***Discussion:*** **Although CMV-positive cases are clinically indistinguishable from CMV-negative cases, our PCR system allowed the retrospective diagnosis of CMV-related SNHL.** ***Conclusion:*** **CMV-related SNHL tends to accompany mental retardation and low birth weight more frequently than does CMV-negative SNHL.** ***Key Words:*** **Cytomegalovirus infection, sensorineural hearing loss, polymerase chain reaction.**
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INTRODUCTION

Sensorineural hearing loss (SNHL) is the most common congenital disease. Much emphasis has been placed on early hearing detection and diagnosis for early speech and language development as well as on the treatment of subsequent problems.[1,2]

Cytomegalovirus (CMV) infection is the most common intrauterine viral infection. Although approximately 90% of congenitally infected infants remain asymptomatic, the remaining 10% develop neurologic deficits, chorioretinitis, or SNHL. SNHL is one of the most frequent manifestations in patients with congenital CMV infection at birth.[3–6] Late onset of SNHL and mental retardation (MR) in many patients with congenital CMV infection who show few clinical manifestations at birth make the problem more complex because determination of the causal relationship with CMV becomes impossible after 3 weeks from birth because of the possibility of postnatal infection.[5–10] However, dried umbilical cord specimens can be used for the retrospective diagnosis of congenital CMV infection.[11] In Japan, obstetric hospitals customarily provide the dried umbilical cord to the parents of newborns as a symbol of the bond between mother and child. Thus, this material is generally stored with great care at home and is available for almost all individuals born in Japan.

It remains unclear whether there are any differences in the clinical characteristics between CMV-related and -unrelated SNHL cases. In this study, we scrutinized the medical records of the SNHL patients whose congenital CMV infection was identified by polymerase chain reaction (PCR) using their dried umbilical cords and compared

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TABLE I.
Profile of Congenital Cytomegalovirus Infection With Sensorineural Hearing Loss (SNHL).

Case	Diagnosis at Birth*	Sex	BW	Age†	ABR‡ (dB)	SNHL Type§	MR	CT Scan	Others
FUK16	(+)	F	2940	3.7	rt(−),lt80	Stable	Severe	Calcification, ventricle dilation	CP, epilepsy
FUK20	(+)	F	2040	5.2	rt90.lt25	Stable	Mild	(−)	
FUK3	(+/−)	F	1816	4.8	rt(−),lt100	Stable	Moderate	Calcification, sylvian fissure dilation	
FUK10	(−)	F	2344	5.7	rt(−),lt(−)	Progressive?	Severe	Brain atrophy, ventricle dilation	CP, epilepsy
FUK31	(−)	M	2995	2.4	rt(−),lt70	Progressive?	(−)	(−)	
FUK19	(−)	M	3006	3.2	rt(−),lt(−)	Stable	Moderate	Slight brain atrophy	
FUK28	(−)	M	3195	1.1	rt(−),lt(−)	Stable	(−)	(−)	

*CMV clinical diagnosis at birth.
†Age at referral to our facilities.
‡ABR (−) means scale out.
§Type of SNHL (progressive or stable) at referral.
BW = birth weight; ABR = auditory brainstem response; MR = mental retardation; CT = computed tomography; + = ; − = ; rt = ; lt = ; CP = cerebral palsy.

them with those of SNHL patients without congenital CMV infection.

MATERIALS AND METHODS

Study Subjects

This study evaluated 31 patients (15 males and 16 females) with SNHL who were referred to the Department of Otolaryngology, the Fukushima Rehabilitation Center and Department of Otolaryngology, Fukushima Medical University School of Medicine, from November 2004 to March 2005, for early speech therapy, external hearing aid, or surgical intervention, including cochlear implant. Their ages at referral ranged from 13 months to 16 years and 3 months (average, 5 yr and 11 mo). Audiologic tests, computed tomographic imaging, and developmental evaluation were performed, if necessary, at the referral.

Congenital CMV infection in seven of these patients was identified by the preparation of DNA specimens from their dried umbilical cords followed by a nested PCR for the CMV glycoprotein H gene [Koyano et al. 2004; Ogawa et al., Fukushima et al., submitted]. Use of their dried umbilical cord samples and medical records was approved by the Ethical Committee on Human Subjects of Fukushima Medical University School of Medicine and was carried out with informed consent from parents.

Audiologic Evaluations

Hearing levels of the patients were measured by at least one of the following objective audiologic tests, auditory brainstem response and auditory steady-state response, as well as by at least one of the following subjective tests, play audiometry, conditioned orientation reflex audiometry, and pure-tone audiometry. All tests were performed by audiologic experts and repeated at least twice to confirm the measurements.

Clinical Characterization

MR was diagnosed on the basis of the basic of standard Japanese developmental examinations such the Tsumori-Inage and Tanaka-Binet tests and the Wecheler Preschool and Primary Scale of Intelligence. Patients were classified into the following four categories of MR based on the development quotient scores from the Tsumori-Inage test: severe (less than 50), moderate (51–70), mild (71–90), and negative (>90). Cerebral palsy, autism, and other multiple disorders were clinically evaluated.

Statistics

The chi-square test was used to evaluate statistical significance.

RESULTS

Clinical Profile of CMV-Related SNHL Cases

Table I summarizes the clinical characteristics of the seven CMV-associated SNHL patients. Congenital CMV infection of FUK16 was originally diagnosed by CMV immunoglobulin (Ig)M positivity at birth. The CMV serologic status of the mother of FUK20 was confirmed to be IgM-positive seroconversion during her pregnancy, and the unborn child experienced intrauterine growth retardation, suggesting congenital CMV infection. However, no confirmatory test, such as CMV culture, was performed at the time of birth. TORCH, (T)oxoplasmosis, (O)ther Agents, (R)ubella, (C)ytomegalovirus, and (H)erpes Simplex, syndrome was diagnosed for FUK3 by a clinician, although the CMV IgM serology was negative, and no culture/PCR was performed.

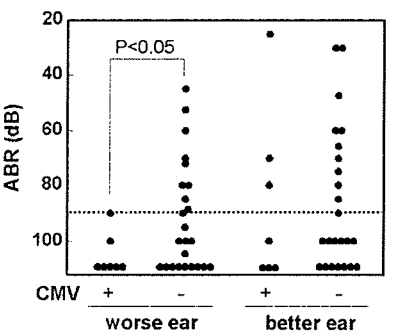

Fig. 1. Hearing levels (dB) of worse and better ears of the 7 cytomegalovirus (CMV)-positive (+) and 24 CMV-negative (−) sensineural hearing loss (SNHL) patients plotted, respectively. Each dot represents one patient. Difference in frequency of severe SNHL, which is defined as >90 dB hearing impairment (dashed line) between CMV-positive and -negative cases was statistically significant ($P < .05$) for worse ear but not for better ear.

TABLE II.
Frequency of Mental Retardation.

	Positive				
	Severe	Moderate	Mild	Negative	Total
CMV (+)	3	1	1	2	7
CMV (−)	0	4	2	18	24

CMV = cytomegalovirus.

Audiologic Findings

Six of the seven CMV-positive cases exhibited profound SNHL (≥100 dB) at the time of referral. Interestingly, some degree of speaking capability in two of the patients, FUK10 and FUK31, was observed until 1 year and 8 months after their birth, suggesting that their SNHL was progressive.

Comparison of hearing levels of the CMV-positive cases with the negative cases shows that CMV-positive cases had more severe hearing impairment in their worse ear (Fig. 1). Frequency of SNHL with more than 90 dB in the worse ear was more frequent in the CMV-related cases than in the unrelated cases ($P < .05$). There was no hearing impairment pattern specific to the CMV-related cases, although several hearing tests were performed (data not shown).

Difference in Frequency of MR Between CMV-Positive and -Negative Cases

MR was diagnosed in 5 of the 7 CMV-positive cases but in only 6 of the 24 CMV-negative cases. Cerebral palsy was diagnosed in only two CMV-positive cases. MR tended to be more prevalent in CMV-positive cases (Table II). In addition to the higher frequency, CMV-positive SNHL cases showed more severe MR.

Lower Birth Body Weight of CMV-Positive Cases

As shown in Figure 2, 3 of the 7 CMV-associated SNHL cases had lower birth body weights for their gestation period than the normal range, whereas those of all 24 CMV-positive cases were in or above the normal range ($P < .01$).

DISCUSSION

This study found that CMV-associated SNHL cases were more frequently accompanied by severe SNHL, MR, and low birth weight than were CMV-unrelated SNHL cases. However, these clinical characteristics are not necessarily exclusive to CMV-related SNHL, and clinical diagnosis of congenital CMV infection in SNHL is almost impossible. Thus, retrospective diagnosis using preserved specimens obtained at birth, such as dried blood spots and dried umbilical cords, is an effective method of clarifying the etiology of SNHL.

Previous studies documented frequent incidences of MR in children with congenital CMV infection.[3,5,7] Evaluation of MR requires careful examination by experts because hearing impairment tends to lower MR scores in SNHL patients. However, it is unlikely that hearing impairment confounded the difference in the prevalence of MR because one SNHL patient population was compared against another SNHL population in this study. In addition, because all 31 patients were evaluated by the same physicians using the same standards and because those physicians were blinded from the laboratory results on congenital CMV infection, it is unlikely that the difference in prevalence of MR between CMV-positive and -negative cases stemmed from subjective bias. Importantly, in addition to the higher prevalence, CMV-positive cases had more severe retardation, supporting the notion that congenital CMV infection causes not only SNHL but also MR. A larger scale study is required to confirm whether our findings can be generalized.

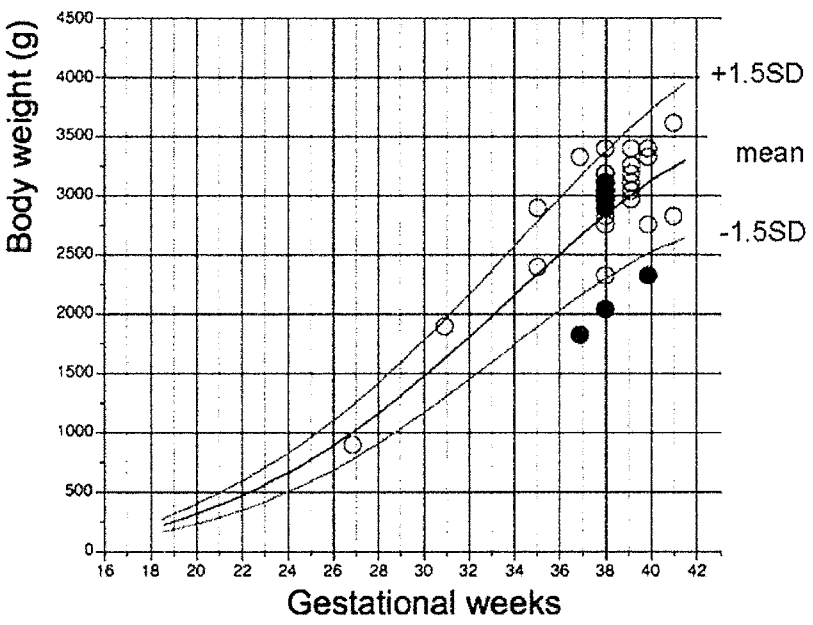

Fig. 2. Birth weights of the cytomegalovirus (CMV)-positive (+) sensineural hearing loss (SNHL) patients (n = 7, closed circles) and CMV-negative (−) SNHL patients (n = 24, open circles) were plotted on the Fetal Growth Standard Curve for Japan.[16]

Several studies indicated that low birth body weight was a major risk factor for HL.[1] However, these studies did not categorize SNHL patients based on known or unknown causes. This study suggests that congenital CMV infection can explain a significant number of SNHL patients with low birth weight.

Several studies that followed up newborns with congenital infection demonstrated late-onset SNHL.[5,9] The identification of two such cases in our study supports the notion that newborn hearing screening examination cannot identify some cases of CMV-related SNHL.[12]

Taken together, we propose the following, as others advocated previously[10,12,13]: 1) to detect SNHL at an early stage of infancy, not only hearing screening but also a CMV screening test is necessary for newborns, and 2) because some cases of CMV-related SNHL progress after birth, it is important to formulate effective hearing examinations and safe anti-CMV treatments. In fact, treatment with ganciclovir in clinical trials was found to delay the progression of SNHL and diseases involving the central nervous system.[14,15]

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Production of Fab Fragment Corresponding to Surface Protein Antigen of *Streptococcus mutans* Serotype c-Derived Peptide by *Escherichia coli* and Cultured Tobacco Cells

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The cDNA of a mouse Fab fragment was cloned from a hybridoma cell line that produces a mouse monoclonal antibody, KH5, that reacts with the peptide fragment of the surface protein antigen of *Streptococcus mutans* serotype c (PAc). After transfection with cDNA, recombinant Fab fragments were produced by *Escherichia coli* (T15 Fab) and cultured tobacco cells (X253 and X262 Fabs). The antipeptide activities of T15 and X253 were similar to that of KH5. X253 was secreted into the culture media, which had a specific affinity for the PAc peptide.

[**Key words:** recombinant Fab fragments, tobacco cell culture, *Streptococcus mutans*, surface protein antigen of *Streptococcus mutans* serotype c (PAc)]

Streptococcus mutans is thought to be a causative agent of dental caries and its cell surface protein antigen (PAc) has been implicated as a cariogenic factor (1). PAc is a major adhesion molecule of *S. mutans* responsible for colonization on the tooth surface and is an important target for the development of anticaries agents. An anti-PAc antibody has been reported to prevent recolonization by *S. mutans* (2). In addition, PAc peptides have been studied as caries vaccines and found to induce anti-PAc antibodies that inhibit the colonization on the tooth surface by *S. mutans* (3, 4). Several monoclonal antibodies (MAbs), designated as the KH and SH series, have been developed (4) that are candidates for anticaries reagents or caries risk diagnostic tools. KH5 is an IgG1, which is reactive to the PAc (361–377) peptide and one of the most reactive antibodies to recombinant PAc among the KH and SH MAb series.

Past studies of anti-*S. mutans* antibodies were focused on the prevention of recolonization (2). The removal of biofilms on the tooth surface by professional cleaning with chlorhexidine before treatment with a large amount of antibodies was necessary. An *S. mutans*-specific bacteriocide that could penetrate the biofilm on the tooth would prevent dental caries more effectively and be a simple medication. The use of an antibody-based drug delivery system (DDS) is one possible method for the development of *S. mutans*-specific anticaries reagents. An immunoliposome, which is a liposome fused with either single-chain antibodies (scFvs) or Fab fragments, is being studied for cancer therapy (5). Dental biofilms have a complex structure and are relatively impermeable to antimicrobial reagents (6). In addition, reagents are easily washed out from biofilms by saliva. However, small antimicrobial molecules with an affinity to *S. mutans* will work more effectively than reagents with no affinity. Antimicrobial peptides fused with small antibodies can be produced (7). PAc-specific recombinant antibodies can be good tools both for producing DDSs for caries prevention and for the diagnosis of caries risk.

Therapeutic antibodies are usually produced by mammalian cell cultures, and the costs are high. It would not be logical to spend as much on caries prevention as on cancer therapies. Moreover, prophylactic anticaries agents must be safer than therapeutic agents. Plants are inexpensive hosts for antibody production and their use reduces the risk of contamination by human pathogens (8). In this study, we investigated plant cells as practical candidate hosts for anticaries antibody production. We cloned Fab cDNA from a MAb KH5-secreting hybridoma cell line for expression in both *Escherichia coli* and cultured tobacco cells. The anti-PAc activities of Fabs were determined and conditions for expression in tobacco cultures were investigated.

MATERIALS AND METHODS

cDNA cloning of Fab fragments and construction of expression vectors Total RNA was isolated from KH5-secreting hybridoma cells (4) and cDNAs of the Fab heavy (H) and light (L) chains were amplified using an antibody cDNA cloning kit (Immunogene M; Nisshinbo Industries, Tokyo). The cDNAs were ligated into the pFab1-His2 vector (9). The selected cDNAs of the

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A.

pT15: tacSD-pelB-FabL-tacSD-pelB-FabH-6xHis

pBI: RB-NPT II cassette-Fab cassette-LB

p23: 35SPro-FabH-6xHis-NosT-35SPro-FabL-NosT

p24: 35SPro-FabH-6xHis-KDEL-NosT-35SPro-FabL-KDEL-NosT

p25: 35SPro-CalSP-FabH-6xHis-NosT-35SPro-CalSP-FabL-NosT

p26: 35SPro-CalSP-FabH-6xHis-KDEL-NosT-35SPro-CalSP-FabL-KDEL-NosT

B.

pelB signal peptide: MKYLLPTAATGLLLLAAQPAMA
Cal signal peptide: MATQRRANPSSLHLITVFSLLVAVVSA

FabH-6xHis
1:EVHLVESGPGLVAPSQSLSITCTVSGFSLTTYAVHWVRQPPGKGLEWLGIIWPGGNTHYN
61:STLMSRLSISKDDSKSQVFLKMNSLQTDDTAMYYCARVGRDYFDSWGQGTTLTVSSAKTT
121:PPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTL
181:SSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRD-GGGGSHHHHHH

FabL
1:EIVLSQSPAIMSASPGEKVTISCSASSSVSYMYWYQQKPGSSPKPWIYRTSNLASGVPVR
61:FSGSGSGTSYSLTISSMEAEDAATYYCQQYHSYPPTFGGGTKLEIKRADAAPTVSIFPPS
121:SEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTL
181:TKDEYERHNSYTCEATHKTSTSPIVKSFNRNECGAP

FIG. 1. Schematic representation of expression cassettes of Fab genes. (A) pT15 was the *E. coli* expression plasmid for the Fab genes. The cDNAs of the light (Fab L) and heavy (Fab H) chains were cloned under the control of the tac promoter and Shine–Dalgano sequence (tacSD). The periplasmic space secretion sequence of *Erwinia cartovora* (pelB) followed the tacSD sequences. A 6× His tag was attached to the C-terminal of the Fab H chain. Fab genes were cloned into the T-DNA region of the binary vector, which has right (RB) and left border (LB) sequences. The p23 and p24 plasmids have Fab H and L chain cDNAs under the control of the cauliflower mosaic virus 35S promoter and omega sequence (35SPro). The nopaline synthetase terminator (NosT) was located downstream. The p25 and p26 plasmids have the secretion sequence of calreticulin (CalSP) of *Nicotiana plumbaginifolia*. The p24 and p26 plasmids have the KDEL ER retention signal at the 3′ end of the Fab H and L chain cDNAs. (B) Deduced amino acid sequences of pelB and CalSP, Fab H-6x His and Fab L.

Fab H and L chains were cloned into the T-DNA region of the pBI101 binary vector with additional synthesized DNA coding for a signal peptide and an ER retention signal (10) (Fig. 1). Translational enhancers (omega sequence and initiation sequence) were introduced immediately downstream of the 35S promoter, as previously described (10), to enhance Fab production.

Expression in *E. coli* and extraction of soluble Fab fragments The ligated pFab1-His2 vector was transfected into *E. coli* JM109 cells (Toyobo, Osaka). Bacterial colonies were incubated at 28°C in 2 ml of Luria–Bertani (LB)/ampicillin (50 mg/*l*) then isopropyl-β-D-thiogalactopyranoside (IPTG: 0.5 mM) was added to induce the expression of the Fab fragments. *E. coli* pellets were suspended in B-PER PBS (Pierce Biotechnology, Rockford, IL, USA)/Complete (Roche Diagnostics, Tokyo) and the anti-PAc activity of the supernatants was determined by ELISA. Five hundred milliliters of the culture media from a selected bacterial clone centrifuged and the pellet obtained was resuspended in 25 ml of B-PER PBS/Complete/5 mM imidazole (Sigma-Aldrich Japan, Tokyo). The cell extract was centrifuged and the supernatant was filtered (0.45 μm) before liquid chromatography.

Transformation of tobacco cells and extraction of Fab fragments Suspension cultures of XD6S tobacco cells (11) were transformed using the recombinant binary vectors p23, p24, p25 and p26 by standard methods (10). Between 10 and 20 colonies from each of the four transgenic lines, X23–X26 series, were inoculated into the MS medium. The transgenic cells were propagated for 7 d under the same conditions used for XD6S cells (11). To measure both total soluble protein (TSP) concentration and the expression level of the antibody, 1 ml of suspension culture was transferred into a 2-ml microtube. One-tenth milliliters of 10× Complete (Roche) in 0.5 M Tris pH 7.5 and 0.15 g each of 0.1- and 1-mm diameter glass beads (Sigma-Aldrich) were added. The tubes were shaken at 30 Hz for 5 min using the Mixer Mill MM300 (Qiagen, Tokyo) and centrifuged at 15,000 rpm for 10 min to obtain the plant extract supernatants. TSP concentration was determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

For antibody purification, tobacco culture suspensions in multiples of 100-ml volumes were sampled 10 d after the initiation of culture. The cells and media were separated by vacuum filtration through filter paper (Advantec, ToyoRoshi, Tokyo). The protein in the media was concentrated by ammonium sulfate precipitation and the resulting pellet was suspended in phosphate-buffered saline (PBS)/Complete/5 mM imidazole. The suspension was filtered (0.45 μm) before liquid chromatography. Cells were frozen overnight and thawed in 2 volumes (v/v) of extraction buffer (50 mM Tris pH 7.5/200 mM NaCl/Complete/5 mM dithiothreitol [DTT]). The cells were homogenized (Physcotron, Microtech, Tokyo) until over 80% of the cells were disrupted, as determined by microscopic observation. Cell debris was removed by centrifugation at 18,000 rpm for 30 min. The protein in the supernatant was concentrated by ammonium sulfate precipitation and the resulting pellet was suspended in PBS/Complete/5 mM imidazole. The suspension was filtered (0.45 μm) before liquid chromatography.

ELISA ELISA plates (SUMILON H type; Sumitomo Bakelite, Tokyo) were coated with 1 μg/well recombinant PAc isolated from *S. mutans* TK18 as described previously (4). Plates were blocked overnight with 3% skim milk (Wako, Tokyo) in PBS to inhibit nonspecific binding. After washing with PBS containing 0.05% Tween 20 (PBST), 100 μl of either 2-fold serially diluted extracts